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23

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<b>(54) Title:</b> IMMUNOGENS AND IMPROVED METHODS OF MAKING IMMUNOGENS  <b>(57) Abstract</b>  Immunogens and methods of making immunogens capable of inducing active immunity against a given agent. The method comprises generating antibodies against the target cell receptor to which the agent binds. The antibodies which mimic the agent's receptor-specific binding site, can be used as immunogens to elicit antibodies in a host which resemble the receptor's binding site and are capable of binding with the agent's ligand, thereby preventing the binding of that ligand to the target cell receptor.		

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IMMUNOGENS AND IMPROVED  
METHODS OF MAKING IMMUNOGENS

This application is a continuation-in-part of our copending application Serial No. 889,424 filed on July 23, 1986.

TECHNICAL FIELD OF INVENTION

This invention relates to a new type of immunogen and a method of making immunogens which mimic the receptor-specific binding site of a given agent. These immunogens can be used to induce an active immune response against the agent in a host. More specifically, this invention relates to a simplified process for the production of antibodies capable of eliciting an active immune response against a given agent, wherein the host's antibodies block the binding of the agent's ligand to the host's target cell receptor.

BACKGROUND OF THE INVENTION

This invention relates to a method of making immunogens useful in the active immunization of a mammal against a given agent. For example, these immunogens can be

- 2 -

used as vaccines against infection by various pathogens or pathogenic agents. In addition, they can be used as a protection against overproduction or undesired production of hormones or enzymes, as well as against any other agent which possesses a receptor-specific binding site.

Immunogens have generally been associated with vaccination, the principal means by which human and animal populations are protected against infectious diseases. Typically, such immunogens, or vaccines, are composed of the killed or attenuated infectious agent itself, its detoxified products or a purified antigen derived therefrom which retains immunochemical identity with the naturally occurring pathogenic agent.

The traditional method of obtaining an antigen and inducing immunity against it has many associated disadvantages, including the difficulty of purifying antigens. In addition, some attenuated organisms have been known to regain their virulence. Thus, the traditional approach to immunization has not proven to be suitable for the development of clinically effective, economically practical vaccines against all major human and animal pathogens. Furthermore, many agents, against which immunity may be desired, are not antigenic enough to elicit an antibody response. In view of various disadvantages, alternative types of immunogens have been devised.

For example, internal image anti-idiotypic antibodies have been used to elicit an immune response to the agent which

- 3 -

they biochemically "mimic". Internal images are antigenic determinants (i.e., epitopes), expressed on antibody molecules, that are immunochemically similar or identical to the epitopes found on external antigens. The advantages of using internal image immunogens include the relative ease of production through monoclonal-antibody technology, and safety; i.e., they are not capable of causing the disease sought to be avoided or treated. Furthermore, such internal image anti-idiotypic vaccines have much greater specificity than traditional immunogens.

Antibodies elicited in response to immunization with an internal image anti-idiotypic antibody are capable of binding to both the immunogen and to any external antigen expressing the epitope mimicked by the internal image. Under experimental conditions, such immunogens have been shown to elicit protective immune responses against a variety of pathogenic agents [R. C. Kennedy et al., "Vaccines Utilizing Internal Image Anti-Idiotypic Antibodies That Mimic Antigens of Infectious Organisms," Bio-Techniques, 3 p. 404 (1985); R. C. Kennedy et al., "Anti-Idiotypes and Immunity," Scientific American, 255, pp. 48-56 (1986)].

Advantageously, internal image anti-idiotypic antibody immunogens allow for the selective targeting of antigenic structures that have functional roles in the development of diseases or enzymatically or hormonally mediated conditions.

- 4 -

For example, an essential early event in many infections and intoxications consists of the specific association between a receptor-specific antigenic determinant expressed by an agent and a receptor expressed by the target tissue. Antibodies that block this association can interrupt the sequence of events leading to the disease.

Internal image anti-idiotypic antibodies are particularly suited for application as vaccines against pathogens. Internal images of pathogen-associated epitopes known to be involved in target tissue binding can selectively elicit pathogen specific antibodies that block the binding of a pathogen to its target tissue. The effectiveness of this approach has been experimentally demonstrated [A. H. Sharpe et al., "Syngeneic Monoclonal Anti-idiotypic Can Induce Cellular Immunity to Reovirus," J. Exp. Med., 160, p. 1195 (1984)].

The application of this technology to the development of immunogens has been impeded, however, by practical difficulties encountered in the generation of internal image anti-idiotypic antibodies. This is in part due to the laborious process through which internal images have heretofore been obtained. Unlike traditional vaccines, which are derived directly from given agents, internal image anti-idiotypic antibodies have been produced by a two-phase procedure. This involves the production of specific antibodies against a given agent which are then used to elicit anti-idiotypic antibodies,

- 5 -

which must be subsequently assayed for the expression of internal images.

For example, to make an anti-idiotypic antibody which expresses an internal image of an epitope associated with the binding site of an agent's receptor-specific antigenic determinant, a panel of antibodies, that selectively block the binding of the agent to its target tissue must first be obtained. Experimental animals are immunized with the agent or its ligand (if available), and a cell fusion, using antibody producing cells obtained from the immune animals is performed. Each hybrid cell line is then screened for the secretion of monoclonal antibodies capable of blocking the agent - receptor binding.

According to the traditional method, after the hybrid cell lines are established and working quantities of monoclonal antibodies are produced, the second major phase of the process is begun. This second phase entails the immunization of experimental animals with each of the monoclonal antibodies. Then hybrid cell lines which secrete monoclonal anti-idiotypic antibodies that selectively inhibit the binding of the ligand-specific monoclonals to the agent are generated. Within the anti-idiotypic set of antibodies are antibody sub-sets that bind to the antigen binding site of the ligand-specific monoclonals. This subset of anti-idiotypes expresses internal images of the agent's ligand epitopes.

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- 6 -

Disadvantageously, the proportion of anti-idiotypes which express internal images is generally small when compared to the overall number of specificities elicited in an anti-idiotypic response. Many of the anti-idiotypes that do not express internal images will nevertheless be capable of sterically inhibiting the association of the ligand-specific monoclonal antibodies with the ligand. Therefore, each of the blocking anti-idiotypes must be screened for the expression of immunogenic internal images. This is accessed by monitoring their capacity to elicit agent-specific antibodies upon innoculation into experimental animals. When monoclonal anti-idiotypes that express immunogenic internal images are finally identified, they can be incorporated into an immunogen or a vaccine.

Even under ideal conditions, a great deal of effort must be expended to obtain internal image anti-idiotypic antibodies by the traditional method. The principal drawback of the two phase approach is that as the number of agent-specific monoclonals increases, the labor required to produce and screen anti-idiotypes raised against them increases. This disadvantage is compounded by the failure of current technology to provide for fine control over the specificities of antibodies elicited in response to immunization. There is no guarantee that antibodies specific for an agent's receptor-specific binding site will be present

- 7 -

in an anti-ligand response. Consequently, it is likely that ligand-specific monoclonals generated from numerous animals must be used to elicit anti-idiotypic responses.

A similar lack of control over the specificity of anti-idiotypic responses often multiplies the number of attempts required to generate internal images. Moreover, the probability of obtaining antibodies with a desired specificity is very low in each immunization and screening step. Therefore, the "two-step" procedure of making immunogens, substantially decreases the probability of obtaining the desired antibody. The increases in complexity, which are inherent in the two step approach, result in substantial increase in the time and labor required to produce a selected internal image, and a decrease in the amount of internal image antibody produced. In some instances the desired internal image may be altogether impossible to produce according to traditional methods.

#### SUMMARY OF INVENTION

The present invention solves the problems referred to above by providing a method for the direct generation of antibodies which mimic the receptor-specific binding sites expressed by various agents. More specifically, by virtue of this invention one can obtain immunogens or immunogenic compositions by means of simplified process employing a single immunization step against the target cell receptor molecules.

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- 8 -

According to the method of this invention, an antibody response is elicited against the receptor to which a given agent specifically binds. By subjecting the receptor-specific antibodies to the selection process described herein, antibodies are obtained that immunochemically mimic the receptor binding site expressed by the agent's ligand. Such "internal images" can subsequently be incorporated into an immunogen designed to elicit antibodies specific for the agent's receptor binding site which are capable blocking the specific association between the agent, i.e., a pathogen, hormone, enzyme, etc., and its target tissue.

#### DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth:

AGENT - any entity which is capable of binding to a cell-surface receptor. As used in this application, the term "agent" includes but is not limited to pathogens, hormones, enzymes, neuromodulators, neurotoxins, and other cytotoxins.

PATHOGEN, PATHOGENIC AGENT - any disease-causing infectious organism (including virions, bacteria, fungi, protozoa, and multicellular organisms) or toxic substance.

RECEPTOR - the molecular entity expressed on a cell or tissue to which an agent specifically binds.

- 9 -

LIGAND - the molecular entity which is responsible for specific binding of an agent, such as pathogen, to a particular receptor.

TARGET TISSUE - the tissue or cells to which an agent specifically binds in the course of an infective, toxic, hormonally mediated, enzymatically mediated or otherwise mediated process.

INTERNAL IMAGE - an antigenic structure expressed by an antibody molecule, which immunochemically mimics an epitope possessed by an agent.

The present invention relates to immunogens or immunogenic compositions and methods of making these immunogens or immunogenic compositions to be used in the active immunization against various agents. The process comprises obtaining antibodies which mimic an agent's receptor-specific binding sites in a single step. The immunogens are formed by eliciting antibodies against the cell surface-receptor to which the given agent binds. After these antibodies are formed, they are used as immunogens to induce an active immunity in a host against the given agent.

According to the method of this invention, one first generates an antibody response against the receptor to which the agent specifically binds.

If the receptor has been identified, it can be injected in pure form, semi-purified, or in a nonpurified

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- 10 -

state. If the receptor itself is unknown, then the target tissue in general can be used as a source of this first immunogen. In this case, the immunogen can consist of purified or semi-purified compounds extracted from the target tissue, crude fractions of target tissue extracts, cellular fragments, whole cells, or multicellular tissue preparations.

The anti-receptor antibody response can be generated in any commonly targeted species and by any of the standard immunization methods. In a preferred embodiment, mice are immunized intraperitoneally with 100 ugm of receptor-containing cells or tissue emulsified in Freund's Complete Adjuvant, then boosted intraperitoneally at regular three week intervals with 100 ugm doses of the receptor-containing cells or tissue suspended in phosphate-buffered saline solution.

The presence of receptor-specific antibodies in the sera of the immunized animals can be verified by means of any commonly employed antibody detecting assay. Typical tests would include precipitation, agglutination, immunoelectrophoresis, immunoblotting, RIA, ELISA, immunocytochemical staining techniques, or bioassays designed to assess the capacity of the antibodies to bind to the receptor and thereby prevent the association of the agent with the receptor.

Using the receptor-immune mice as antibody producing cell donors, one then performs a cell fusion to create

- 11 -

hybridoma cell lines. Standard cell hybridization procedures may be followed [see e.g., P. B. Mishell and S. M. Shiigi, Selected Methods In Cellular Immunology, pp. 351-71, San Francisco (1980)].

The next step involves screening the hybridoma cells for the production of antibodies that specifically block the association of the agent with its receptor. According to one embodiment of this invention, the screening procedure may employ an assay system capable of detecting the inhibition of agent binding by the receptor-specific antibodies including, but not limited to, various immunocytochemical techniques, autoradiography, precipitation, and agglutination assays.

After cell lines are established, practical quantities of monoclonal antibodies may be produced using established procedures [Mishell, supra].

The next step, involves a determination of whether the receptor-specific monoclonal antibodies express an internal image of the agent's receptor binding site. Because numerous receptor-specific monoclonal antibodies will likely have been identified, it is preferable that a method be used to determine which of the monoclonal antibodies are more likely to express an internal image. This method can consist of any assay capable of detecting epitopes expressed by the monoclonal antibodies that mimic epitopes expressed by the agent. For example, this initial screening step could be made by assaying

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- 12 -

the binding of the receptor-specific antibodies to agent-specific antibodies, obtained from animals immunized against the agent. Receptor-specific monoclonal antibodies that express internal images of the agent's receptor binding site would bind to agent-specific antibodies directed against the agent's receptor binding site.

The receptor-specific antibodies would next be subjected to the final and definitive selection procedure. In this procedure the receptor-specific antibodies are used to immunize experimental animals, and the resultant antisera are screened for the presence of agent-specific antibodies that are capable of blocking the binding of the agent and its target tissue. Standard animal species and immunization procedures may be employed. For example, rabbits may be injected subcutaneously with 100 ugm of monoclonal antibody absorbed to alum or emulsified in Freund's Complete Adjuvant, then boosted subcutaneously with 100 ugm doses administered at three-week intervals. Agent-specific blocking antibodies may be detected using the assay used in the screening step, above. However, in this case, the agent (rather than the receptor) is treated with the antisera prior to exposure to the target tissue.

Internal images thus identified can subsequently be subjected to vaccine testing trials.

The preferred embodiment of this invention results in the generation of monoclonal internal image secreting cell

- 13 -

lines; however, the same basic approach may be utilized for the production of internal images from serum. In such instances, it would be preferable to immunize larger species (such as goats, pigs, or cattle) against the receptor because larger animals yield greater quantities of antibody on a per animal basis.

The principal difference between this invention and the standard method of obtaining internal images is that this technique is based upon the generation of antibodies against the receptor to which an agent specifically binds. As described above, this is a substantial improvement over the standard procedure which relies upon the generation of anti-idiotypic antibodies from previously generated agent-specific antibodies. According to the methods of the prior art, antibodies against the given agent's binding site would first have to be formed. Then, those antibodies would have to be used to elicit other antibodies before a vaccine could be made which induces active immunity against the agent's binding site. Clearly, the present invention provides a superior approach because it avoids the increase in labor and time imposed by the first of two immunization series. Furthermore, it increases the probability of obtaining the desired internal image antibody.

The method of this invention can be applied to the development of internal image immunogens that block the binding



- 14 -

of virtually any ligand molecule for which a receptor exists. It is not necessary that the receptor be specifically identified if the target tissue which expresses the receptor is known. Potential targets include the receptor binding sites of ligands expressed by toxins, virions, bacteria, fungi, protozoans, and multicellular parasites.

For example, the internal images of the T-4 binding site expressed by HTLV-III, the causative agent of AIDS, may be produced by immunization with the T-4 antigen found on the human T-cell subset targeted by HTLV-III. The HTLV-III ligand internal images may subsequently be incorporated into a vaccine designed to elicit antibodies capable of blocking binding of the virus to T-4 cells. Thus, T-4 cells are protected from HTLV-III infection, because the ligand-specific antibodies prevent the virions from binding to the cells.

This invention includes immunogens which can block other receptor-ligand binding, exemplified by, but not limited to, the binding of hormone ligands, ligands that bind to antigen receptors expressed by B-cells and T-cells, as well as ligands that bind to the catalytic sites of enzymes.

A major advantage of this invention is that it provides immunogens which mimic genetically stable epitopes. In one embodiment of the present invention, immunogens are used to make vaccines against pathogens. Many pathogens are capable of continually infecting potential hosts because of "antigenic

- 15 -

drift", brought about by selective pressure resulting from increasing levels of immunity in once susceptible host populations. Immunogens which elicit immunity against pathogenic epitopes subject to antigenic drift, may be rendered ineffective as the epitopes change with time.

However, not all of a given agent's antigenic structures are subject to the same degree of mutational freedom; certain structures, such as those which provide vital functions in a pathogen, cannot be altered to the extent that their physiological role is lost. Included within this category of antigenic determinants are the structures responsible for the specific binding of pathogens to receptors expressed by target host cells. The importance of target cell binding in the infectious process mandates that the pathogen's epitopes which are involved in binding undergo few modifications if any. If any modification in the pathogen's epitope occurred the capacity to bind target cell receptors could be lost.

The present invention specifically targets these genetically stable epitopes, to produce immunogens that are minimally affected by antigenic drift. From a practical perspective, this approach provides an extraordinary advantage over immunogens that target epitopes subject to antigenic drift.

Through the careful selection of internal image antibody specificity, injection, dose, route of administration,

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- 16 -

adjuvant and vehicle, and internal image isotype, this invention provides safe, effective immunogens which avoid the induction of autoimmune disease or other pathogenic processes that otherwise could result from the injection of antibodies that bind to normal body antigens.

Administration of these immunogens, compositions containing them, or pharmaceutically acceptable and immunologically effective derivatives thereof, should be via any of the conventionally accepted modes of administration of agents which exhibit immunogenicity. These include oral or parenteral administration.

The compositions used in these vaccines may be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as powders, liquid solutions or suspensions, suppositories, injectable and infusable solutions. The preferred form depends on the intended mode of administration and therapeutic application.

The compositions also will preferably include conventional pharmaceutically acceptable carriers and may include other medicinal agents, carriers, adjuvants, excipients, etc., e.g., human serum albumin or plasma preparations. Preferably, the compositions of the invention are in the form of a unit dose. The amount of active compound administered as a vaccination or as a medicament at one time, or over a period of time, will depend on the subject being

- 17 -

treated, the manner and form of administration, and the judgment of the treating physician. However, an effective dose may be in the range of from about 1 ng to about 1 mg of the composition of this invention, preferably about 100 ug to about 500 ug; it being recognized that lower and higher doses may also be useful.

Accordingly, this invention provides immunogens and methods of making immunogens. Such immunogens are capable of eliciting an active immune response against a given agent, in mammals, including humans.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only, and are not to be construed as limiting this invention in any manner.

#### EXAMPLE 1

In this example we demonstrate a means by which the method of this invention could be used to produce a vaccine capable of eliciting a protective antibody response against Venezuelan Equine Encephalomyelitis Virus (VEEV). In broad outline, mice are initially immunized against cells targeted by VEEV. After demonstrating that the anti-target cell antibody responses block VEEV infection of cultured cells, a cell fusion would be performed to generate monoclonal antibodies which

- 18 -

mimic the virus' (VEEV) receptor-specific binding site. These monoclonal antibodies are identified by their capacity to prevent VEEV infection of susceptible cells in vitro. Next the monoclonals would be subjected to a second selection procedure to assess their capacity to elicit antibodies that bind to VEEV. This step identifies receptor-specific monoclonal antibodies that can be used as immunogens to evoke the formation of antibodies which resemble the target cell's receptor for VEEV and which bind to and neutralize the virus. Finally, both in vitro and in vivo methods would be used to demonstrate that the antibodies elicited by the anti-receptor monoclonals are protective against VEEV infection.

I. IMMUNIZATION OF MICE WITH  
VEEV-SUSCEPTIBLE CELLS

We immunize mice with VEEV-susceptible target cells. We grow BHK-21 cells (Baby Hamster Kidney cells; ATCC, Rockland, Md.) and Vero cells (human cells susceptible to VEEV; ATCC, Rockland, Md.) in M199 culture media (Gibco Laboratories) supplemented with 2% fetal calf serum (FCS) containing 50 ug/ml gentamycin. Then we immunize 12 week old CAF<sub>1</sub> female mice with BHK-21 cells. We harvest the BHK-21 cells from tissue culture flasks and wash them 4 times by centrifugation (400 x g, 5 min.) in FTA hemagglutination buffer (FTA). We resuspend the cells in FTA to  $2 \times 10^7$  cells/ml. After obtaining

- 19 -

pre-immune (normal) serum samples by tail vein bleeding, we innoculate each mouse intraperitoneally with  $10^7$  BHK-21 cells in 0.5ml FTA. We similarly boost the mice twice, at 21 day intervals. Fourteen days after the third injection, we obtain serum samples via tail vein bleeding.

## II. ASSAY FOR SERUM ANTI-VIRAL RECEPTOR ANTIBODIES

In our assay for serum anti-viral receptor antibodies, we use TC-83 (Trinidad Donkey) vaccine strain VEEV [T. O. Berge et al., "Attenuation of Venezuelan Equine Encephalomyelitis Virus By In Vitro Cultivation In Guinea Pig Heart Cells," Am. J. of Hygiene, 73, p. 209 (1961)]. We use a modification of the standard viral plaque reduction neutralization method (PRNM) described in the Pan American Sanitary Bureau publication "International Conference on Equine Encephalitis Vaccine" (1974) to assay for antibodies capable of blocking the binding of the TC-83 vaccine strain VEEV to Vero cells.

We grow monolayers of Vero cells in 100mm culture dishes, and then wash them 4 times with M199 nutrient medium. We dilute the pre-immune and immune sera samples, which we obtain from BHK-21 immunized mice, 1:10 and 1:50 in M199. We add 2.0 ml of each sera dilution to the Vero cell cultures and incubate the cells with the diluted sera for 1 hour at 37° C under 5% CO<sub>2</sub>.

- 20 -

Next, we add approximately 50 plaque-forming units (pfu) of TC-83 virus in 2.0ml of M199 (supplemented with 2% FCS and 50 ug/ml gentamycin) to each culture dish. The cells are incubated in the presence of the virus plus antibody for 1 hour at 37° C under 5% CO<sub>2</sub>.

After incubation, we count the viral plaques. Immune sera that causes a reduction in plaque number equal to or exceeding 50% (in comparison with the number of plaques on the plate treated with pre-immune sera) are considered to contain antibodies specific for the viral receptor expressed on the surface of the Vero cells.

### III. GENERATION OF ANTI-RECEPTOR MONOCLONAL ANTIBODIES

We boost the mice which have been identified by the PRNM as having mounted receptor specific antibody responses for a fourth time, with BHK-21 cells, as previously described.

Three days later, we obtain spleen cells from these mice and fuse them with P3 myeloma cells by the methods described in B. B. Mishell and S. M. Shiigi [Selected-Methods In Cellular Immunology (1980)]. The culture medium consists of RPMI 1640 (Gibco Laboratory) supplemented with 10% FCS and 50 ug/ml gentamycin.

To limit the number of hybrid supernatants which must be screened via the PRNM, we first screen the supernatants for antibodies specific for antigens expressed on the surface of

- 21 -

Vero cell by means of the following ELISA. We rinse the Vero cell monolayers grown in 96 well microtiter plates 4 times with RPMI 1640. We add 100 ul of hybridoma cell culture supernatant to the Vero cell monolayers and incubate the plates for 1 hour at 4° C. Next we add to each well 100 ul of peroxidase-conjugated Rabbit anti-Mouse Immunoglobulin which has been diluted 1:1000 in FTA plus 5% bovine serum albumin. We incubate the wells for 1 hour at 4° C, and then rinse each well 3 times with FTA at 4° C. Next, we add 100 ul of peroxidase substrate per well and incubate the plates for 30 minutes at 22° C. We transfer 50 ul from each well to corresponding wells in Immulon U Plates, and determine absorbance values on a Microelisa Reader. We thereby identify hybrid supernatants which contain antibodies specific for cell surface antigens.

To identify the ELISA-positive hybridomas which produce antibodies directed against the VEEV receptor, we test supernatants in a PRNM. This is done as previously described, except that supernatants from hybridoma cultures are substituted for the mouse sera tested earlier. We then use hybrids which produce antibodies that block TC-83 VEEV infection of Vero cells, to produce monoclonal receptor-specific antibodies as ascites antibody.



- 22 -

IV. IDENTIFICATION OF RECEPTOR-SPECIFIC  
ANTIBODIES THAT INDUCE ANTIBODIES  
WHICH RESEMBLE THE RECEPTOR AND THUS  
BIND THE VIRUS

We next assess the capacity of the receptor specific monoclonals to elicit anti-viral antibodies in mice, as follows.

We render the receptor-specific monoclonals immunogenic for mice by coupling them to keyhole limpet hemocyanin (KLH). This saves us the necessity of using a different species to generate an antibody response to the monoclonals. The monoclonal antibodies are precipitated from ascites fluid by the addition of saturated ammonium sulfate to 40% saturation, and then dialysed against saline. We couple 5.0mg of monoclonal to 10mg KLH in 4.0ml saline by adding 50mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The reaction mix is stirred for 12 hours at 22° C, and we then dialyse it against FTA at 4° C.

We immunize mice with 100 ug anti-receptor monoclonal-KLH conjugates emulsified 1:1 in Freund's Complete Adjuvant H37Ra (Difco). To do this we inject 0.1ml of emulsion intraperitoneally in each mouse. The animals receive two additional intraperitoneal injections of 100 ug of antigen each, administered in FTA at 21 day intervals. We obtain serum samples by tail vein bleedings 14 days after the third injection.

We then assay for pre-immune and immune sera for the presence of VEEV-specific antibodies by ELISA. TC-83 VEEV is

- 23 -

suspended to a concentration of 20 ug of protein per ml in glycine buffer (0.1 M, PH=9.5) and added to Immulon U plate wells at 25 ul/well. After an overnight incubation at 4° C., we wash the plates 4 times with saline, containing 0.05% Tween-20 plus 0.02%  $\text{NaN}_3$ .

We serially dilute the serum samples in ten-fold increments, from  $10^{-1}$  through  $10^{-8}$ . The dilutions are made in a diluting buffer consisting of FTA (supplemented with 0.05% Tween-20 and 0.02%  $\text{NaN}_3$ ). We add 25 ul of each sera dilution to the antigen-coated u-plate wells and incubate for one hour at 22°C. The wells are then washed 4 times. We then add 25 ul of a 1:1000 dilution of Biotin-Rabbit anti-Mouse Immunoglobulin to each well. Following a 1 hour incubation at 22° C, we wash the wells 4 times. We add 25 ul of 1:1000 dilution of avidin-alkaline phosphatase to each well and incubate for 1/2 hour at 22° C. We then wash the wells 4 times and add to each well 25 ul of p-nitrophenylphosphate at 1mg/ml in 10% diethanolamine plus 0.05 mM  $\text{MgCl}_2$  plus 0.02%  $\text{NaN}_3$  at pH = 9.8. After a 20 minute incubation at 22° C, we determine the absorbance values 20 a Microelisa Reader. Immune sera which show a 100 fold increase in anti-VEEV over pre-immune sera are considered to be positive for anti-VEEV antibodies.

- 24 -

V. DEMONSTRATION THAT THE ANTIBODY  
RESPONSE ELICITED BY IMMUNIZATION  
WITH RECEPTOR-SPECIFIC MONOCLONALS  
IS PROTECTIVE AGAINST VEEV

A. In Vitro

We dilute sera from mice immunized against receptor-specific monoclonal-KLH conjugates (as above) in two-fold serial increments from 1:2 through 1:32 in M199. As a control, we use sera from mice immunized with KLH coupled to monoclonal antibodies specific for an unrelated antigen, such as hen egg lysozyme. We mix 2.0 ml of each dilution with 2.0 ml of virulent, wild type VEEV strain to give a final VEEV concentration of approximately 25pfu/ml, and incubate for 1 hour at 37° C.

We next add 2.0 ml of each mixture to washed Vero cell monolayers in 100mm culture dishes. After incubating for 1 hour at 37° C under 5% CO<sub>2</sub>, we rinse the Vero cell monolayers 4 times with M199.

Next, we overlay the Vero cells with 4.0 ml of agar overlay medium and incubate for 48 to 72 hours at 37° C under 5% CO<sub>2</sub>.

After incubation, we count viral plaques. Antisera that causes a 50% reduction in the number of plaques, in comparison with pre-immune sera and non-specifically immunized controls, are considered to be protective against VEEV infection.

- 25 -

### B. In vivo

We immunize mice against KLH-coupled receptor specific monoclonals and KLH-coupled non-specific monoclonals, as set forth above. Ten days after the third inoculation, we inject the mice intraperitoneally with 100 intraperitoneal LD<sub>50</sub> (that dose which kills 50% of the animals injected) of a virulent wild type strain of VEEV. Additional controls are similarly inoculated with TC-83 VEEV.

We then assess mortality for 14 days. A survival rate of 80% is indicative of protection.

### EXAMPLE 2

In this example we demonstrate that immunization against the idiotype of a monoclonal antibody, specific for a cell surface receptor antigen, does not disrupt the normal functions of the cell expressing that receptor antigen.

In this experiment we selected a system involving the immunization of mice with rat monoclonal antibody (Becton Dickinson) specific for Lyt-1. Lyt-1 is an antigenic structure expressed on the surface of murine helper T-cells which can be likened to a cell receptor. Treatment of mice with large quantities of soluble anti-Lyt-1 antibodies can functionally deplete helper T-cell activity. This depletion is demonstrable as a diminution in antibody responses against T-dependent antigens. In the present experiment, we assessed the effects

- 26 -

of immunogenic quantities and forms of rat anti-Lyt-1 monoclonal antibodies upon antibody responses against two T-dependent antigens.

Monoclonal rat antibodies specific for the Lyt-1 surface antigens expressed by murine T-cells were used to elicit idiotype specific antibodies without affecting helper T-cell dependant antibody responses against other antigens. Our results, as shown below, indicate that an appropriately selected, receptor-specific monoclonal antibody can be used to elicit an anti-idiotypic immune response without altering the normal functions of the cell which expresses the specified receptor.

We immunized four month old female CAF<sub>1</sub> mice with either hen egg lysozyme (Miles Scientific), rat anti-Lyt-1 monoclonal antibodies (IgG2a, derived from LOU-Wsl/M rat strain) (Becton Dickinson Monoclonals, Inc.), or normal rat immunoglobulin. The normal rat immunoglobulin consisted of LOU-Wsl/M normal rat sera (Charles River Labs) mixed 1:1 with normal Sprague-Dawley rat sera (Rockland Scientific) that was precipitated with 40% ammonium sulphate then dialyzed against FTA hemagglutination buffer.

Ten mice were injected intraperitoneally with 25 g of rat anti-Lyt-1 antibody absorbed onto 0.1 ml of alhydrogel (Superfos, Ltd.). These ten mice were then boosted twice, with 25 ug of rat anti-Lyt-1 antibody in saline, administered

- 27 -

intraperitoneally. The two boosts occurred at 21 day intervals. We also immunized ten control mice with normal rat immunoglobulin using a protocol identical to that used for the mice receiving rat anti-Lyt-1.

Five mice from each of the above groups were additionally immunized against lysozyme. We administered primary injections of 25 ug lysozyme absorbed onto 0.1 ml of alhydrogel intraperitoneally, 7 days after the first injection of rat antibodies. One boost of lysozyme in saline was administered intraperitoneally 21 days later. Epinephrine (0.1 ml, 1:5000) was injected intraperitoneally with each antigenic (saline) boost.

To follow serum antibody responses, serum samples were obtained from tail vein bleedings taken at 7 day intervals over the course of the experiment.

In order to ascertain whether the mice were producing anti-lysozyme antibodies, anti-rat immunoglobulin antibodies, or anti-rat anti-Lyt-1-idiotypic-specific antibodies we conducted an Enzyme-Linked Immunosorbent Assay ("ELISA"). In this assay, the antibody is labelled by a covalently attached enzyme (instead of the radiolabel used in a radioimmunoassay). The enzyme attached to the antibody is one that can react with a colorless substrate to give a colored product. In an ELISA Assay, the amount of product released in a fixed period of time depends on the concentration of enzyme, and this in turn is a

- 28 -

measure of the amount of antibody present. Specialized spectrophotometric equipment is then used to read the optical densities (O.D.), which correlate with the amount of bound antibody.

We established our ELISA assay, as follows:

we coated Immulon (made by Dynatech) II U-Plate wells with 25 ul of antigen (lysozyme, rat immunoglobulin, or rat anti-Lyt-1 monoclonal antibody) in a solution of coating buffer (0.1M glycine, pH = 9.5) at 10 ug/ml and maintained the coated wells overnight at 4° C. We rinsed the wells four times with a wash solution of saline to which we had added 0.5% Tween-20 and 0.02%  $\text{NaN}_3$ .

To test sera for the presence of antibodies specific for the antigens coating the U-plate wells, we added 25 ul of sera (typically diluted in ten-fold serial increments running from  $10^{-2}$  through  $10^{-8}$  in FTA Hemagglutination Buffer containing 0.05% Tween-20 and 0.02%  $\text{NaN}_3$ ) per well and then incubated the wells for one hour at 22° C. We tested the sera ("primary antibodies") for the presence of anti-idiotypic antibodies in wells coated with rat-anti-Lyt-1 antibodies. In order to do this, we pre-absorbed the sera with normal rat sera at a concentration of 1 mg/ml for one hour at 22° C prior to adding it to the U-plate wells. After the primary antibodies' incubation period had expired, we then rinsed the wells again.

We then added 25 ul per well of secondary antibodies, consisting of biotinylated rabbit anti-mouse Ig (1:1000, in FTA

- 29 -

Hemagglutination Buffer + 0.05% Tween-20 + 0.02%  $\text{NaN}_3$  + 5% normal rat immunoglobulin). We then incubated the wells for one hour at 22° C. We again rinsed the wells. After adding to each well 25 ul of avidin-alkaline phosphatase conjugate (1:1000) we incubated the wells for one hour at 22° C. We again rinsed the wells, and we then added to each well, 25 ul of 1 mg/ml p-nitrophenyl-phosphate (in 10% diethanolamine, 0.5mM  $\text{MgCl}_2$ , 0.02%  $\text{NaN}_3$ , pH = 9.8) and allowed the color to develop for 5-30 minutes. We determined the optical densities with a Dynatech Microelisa Reader.

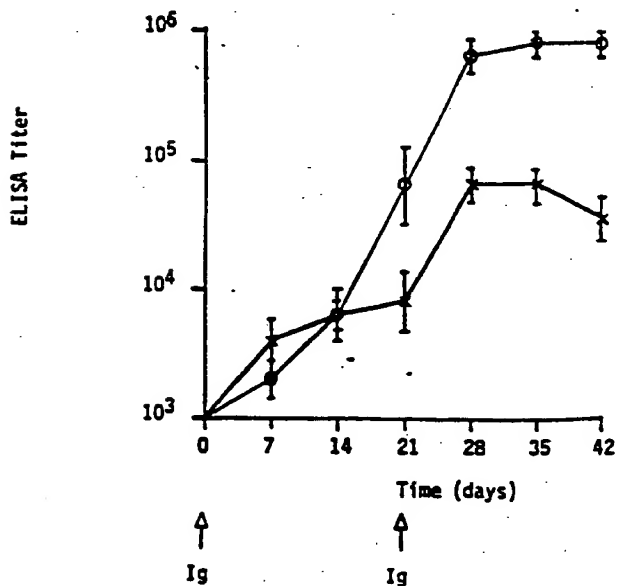
Referring now to Table 1, we have depicted therein the murine anti-rat immunoglobulin response following immunization with rat anti-Lyt-1 monoclonal antibody.



30

Table 1

Anti-Normal Rat Immunoglobulin Antibody  
Titers in Mice Immunized with Rat anti-Lyt-1 (x)  
and Normal Rat Immunoglobulin (o)



n = 10 mice 1 group

Values plotted are means of individual titers,  $\pm$  standard error.

Arrows indicated the days on which rat antibodies (Ig) were administered.

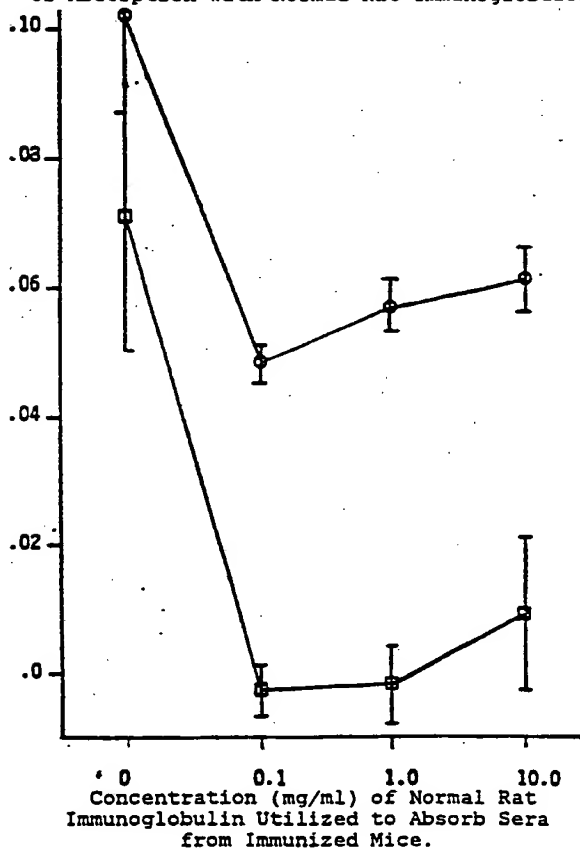
- 31 -

As can be seen, two injections of rat anti-Lyt-1 antibody ("depicted as "Ig" in Table 1) induced anti-rat immunoglobulin antibody titers that approached  $10^5$ . The control animals which had been immunized with normal rat immunoglobulin, responded with titers approaching  $10^6$ . The 10-fold difference between the two groups can be explained in terms of the heterogeneity of the antigen preparations with which each group was injected. The normal rat immunoglobulin preparation is of much greater heterogeneity than the monoclonal antibody. The normal rat immunoglobulin, thus, evokes a more heterogeneous response. This is detected as a higher titer when the immune sera is assayed for activity against normal rat immunoglobulin.

Referring now to Table 2, we have depicted therein the effect of absorption with normal rat immunoglobulin. When the sera were assayed for binding activity against rat anti-Lyt-1 monoclonal antibody, it was apparent that the anti-IgG2a responses elicited in both groups were essentially the same. This is depicted by the data points corresponding to sera absorbed with 0 mg/ml of normal rat immunoglobulin. Our results confirm that the anti-Lyt-1 antibodies did not alter the anti-immunoglobulin response normally elicited under these conditions.

Table 2

Anti-(Rat anti-Lyt-1) Antibody Titers in sera of Mice Immunized with Rat anti-Lyt-1 Antibody (o) or with Normal Rat Immunoglobulin (□): Effect of Absorption with Normal Rat Immunoglobulin.



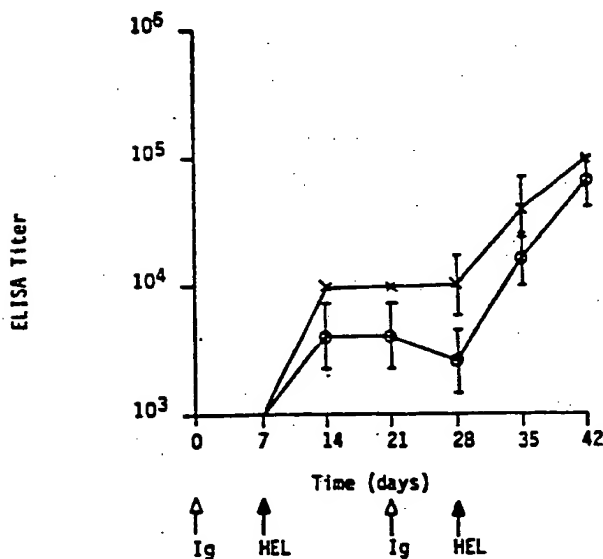
Sera obtained on days 0 and 63 were absorbed with normal rat immunoglobulin then added at a final dilution of  $2 \times 10^{-3}$  to wells coated with Rat anti-Lyt-1 monoclonal antibody. (Quadruplicate samples.) Absorbance values plotted are the means ( $\pm$ s.e.) for sera obtained on day 63 minus the means for sera obtained on day 0.

- 33 -

We then examined the effect of rat anti-Lyt-1 antibody immunization upon the humoral response against an unrelated antigen. As indicated in Table 3, there was no significant difference in the anti-lysozyme antibody responses generated by mice immunized with rat anti-Lyt-1 antibody and those injected with normal rat immunoglobulin. The anti-rat immunoglobulin response shown in Tables 1 and 2, indicates that the animals were capable of responding against T-dependent antigens at the time of anti-Lyt-1 administration. The failure of the rat anti-Lyt-1 antibodies to diminish mouse antibody production against either rat immunoglobulin or lysozyme indicates that immunization with anti-Lyt-1 antibodies does not inhibit the normal helper functions of Lyt1<sup>+</sup> T-cells.

Table 3

Anti-Lysozyme Antibody Titers in Mice Immunized with Lysozyme (HEL) plus Rat anti-Lyt-1 (x) or HEL plus Normal Rat Immunoglobulin (o)



n = 5 mice/group

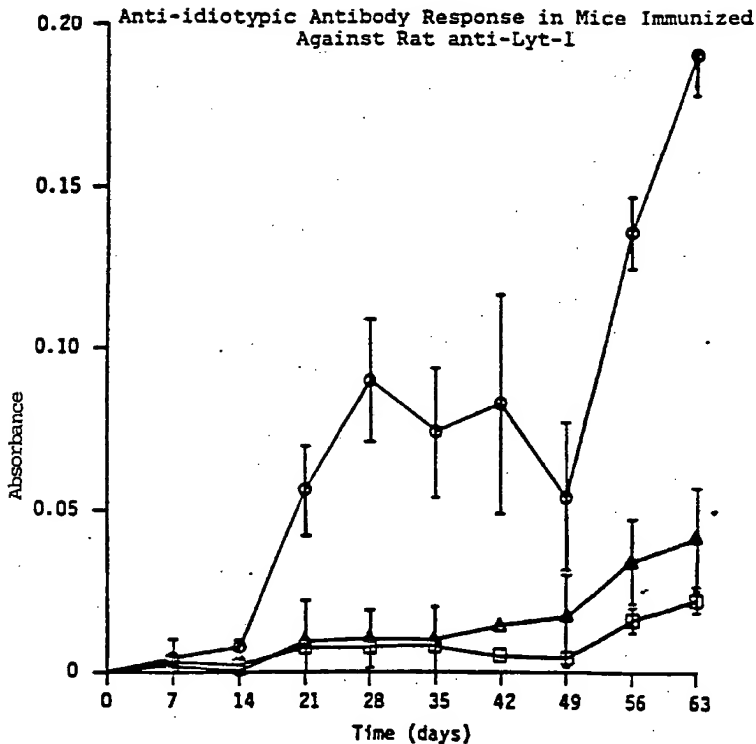
Values plotted are means of individual titers,  $\pm$  standard error.

Arrows indicate the days on which the indicated antigen was injected.

- 35 -

To demonstrate that the immunization protocol had elicited the specific anti-idiotypic response we were seeking, we used normal rat sera to absorb sera obtained from mice which had been immunized against the rat monoclonal antibody and from mice which had been injected with normal rat immunoglobulin. We then assayed for the presence of antibodies specific for rat anti-Lyt-1 antibody.

Table 4



△  
Ag

△  
Ag

△  
Ag

○ : mice immunized against rat anti-Lyt-1, vs anti-lyt-1 (as Ag in ELISA)

□ : mice immunized against rat anti-Lyt-1, vs normal rat; immunoglobulin

△ : mice immunized against normal rat immunoglobulin, vs rat anti-Lyt-1

n = 5 mice/group. ELISA Absorbance values plotted are means minus the background absorbance obtained for sera obtained on day 0. All sera preabsorbed with normal rat immunoglobulin. Values were obtained at  $10^{-3}$  dilution of sera ( $\pm$  standard error).

- 37 -

As shown in Table 4, three injections of the rat anti-Lyt-1 preparation induced an anti-idiotypic antibody response. Binding against normal rat immunoglobulin was negligible. This proved the idiotypic specificity of the response. We did observe a slight increase in binding by sera obtained on days 56 and 63 from mice immunized with normal rat immunoglobulin. This was due to incomplete absorption by the normal rat immunoglobulin, as we verified by absorbing sera obtained on day 63 with various concentrations of normal rat sera prior to assaying for anti -(rat anti-Lyt-1) activity. As we depicted above, in Table 2, any binding by sera from normal rat immunoglobulin-immunized mice was eliminated by absorption. This absorption also neutralized some non-idiotypic specific activity present in the rat anti-Lyt-1 immune sera. Our results thus indicated the anti-idiotypic fraction of the immune response.

Finally, we conducted an immunocytofluorescence assay to demonstrate that the mouse anti-idiotypic sera we had produced was capable of blocking the binding of the rat anti-Lyt-1 monoclonal antibodies to Lyt-1 expressed on the surface of murine thymocytes.

We obtained murine anti-idiotypic antisera from rat anti-Lyt-1 immunized mice by tail vein bleeding on day 42 of the immunization schedule (see discussion above regarding Table 1). We obtained the control sera similarly, from the mice

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- 38 -

immunized with normal rat immunoglobulins. Both antisera preparations were pre-absorbed with normal rat immunoglobulin to remove antibodies specific for rat immunoglobulin determinants other than the anti-Lyt-1 idiotoxes. This was done by diluting each sera 1:100 in FTA hemagglutination buffer containing 0.02% sodium azide (FTA/N<sub>3</sub>), then mixing 0.5ml of each diluted sera with 0.5ml of 0.25mg/ml normal rat immunoglobulin (in FTA/N<sub>3</sub>). The absorption step was allowed to proceed for 1 hour at 0° C.

Next, we reacted the absorbed sera with rat anti-Lyt-1 monoclonal antibodies by mixing 0.5ml of each sera with 0.5 ml of rat anti-Lyt-1 prediluted 1:1000 in FTA/N<sub>3</sub>. As a negative staining control, we mixed 0.5ml of 0.002mg/ml solution of normal rat immunoglobulins with 0.5ml of the absorbed anti-idiotype sera. The reactions were allowed to proceed for 1 hour at 0°C.

We next reacted each of three mixtures with murine thymocytes. To prepare the thymocytes, we obtained thymii from CO<sub>2</sub>-euthanized CAF<sub>1</sub> mice and freed them of lymph nodes. We then minced the thymii in FTA/N<sub>3</sub> at 0° C. After the debris was allowed to settle for 3 minutes, the suspended cells were washed twice in FTA/N<sub>3</sub> and centrifuged (400xg, 5 min.). After a final centrifugation, 5x10<sup>6</sup> thymocytes were suspended in 1.0ml of each of the three antisera mixtures and mixed on a Bellco rocker platform at medium speed for 1 hour at 4° C.

- 39 -

The cells were next washed twice in FTA/N<sub>3</sub> by centrifugation (400xg, 5 min) and then resuspended in 1.0ml of fluoresceinated-Goat anti-Rat Immunoglobulin antibodies (purchased from Antibodies, Inc., Davis, California). We then diluted the cells, 1:100 in FTA/N<sub>3</sub>. The cell suspensions were mixed on a rocker platform for 1 hour at 4° C.

Finally, we washed the cells twice by centrifugation and resuspended them for a concentration of 10<sup>7</sup> thymocytes/ml in FTA/N<sub>3</sub>. We examined the cells for fluorescent staining under a Leitz Laborlux 12 microscope.

Referring now to Table 5, we depict therein the results of our assay:

- 40 -

Table 5

Effect of Anti-(Rat Anti-Lyt-1) or Anti-(Normal Rat Immunoglobulin) Upon The Binding of Rat Anti-Lyt-1 or Normal Rat Immunoglobulins To Mouse Thymocytes As Assessed By Fluoresceinated Goat Anti-Rat Antibodies

<u>Antibody Treatment</u>		
<u>Rat Antibodies</u>	<u>Mouse Antibodies to Rat Antibodies</u>	<u>Fluorescence on Mouse Thymocytes**</u>
Rat Anti-Lyt-1	Mouse Anti-Normal Rat Ig*	+3
Rat Anti-Lyt-1	Mouse Anti-(Rat Anti-Lyt-1)	0
Normal Rat Ig	Mouse Anti-(Rat Anti-Lyt-1)	0
Rat Anti-Lyt-1	none	+3

\* Ig = immunoglobulins

\*\* Fluorescence:

0: no fluorescence  
 +1: very weakly fluorescent  
 +2: moderately fluorescent  
 +3: strongly fluorescent  
 +4: very strongly fluorescent

We were able to discern no fluorescence on thymocytes which had been exposed to rat anti-Lyt-1 monoclonal antibodies and had been mixed with mouse anti-idiotypic sera. Positive

- 41 -

control cells, which had been exposed to rat anti-Lyt-1 mixed with mouse anti-rat immunoglobulins, fluoresced brightly. We detected no nonspecific fluorescence in the negative controls.

Thus, our results show that the anti-idiotypic antibodies were capable of inhibiting the binding of the rat antibodies to Lyt-1. Our results confirm that immunization with receptor specific antibodies can evoke an anti-idiotypic antibody response that functionally neutralizes the receptor-specific binding capacity of the immunogen.

### EXAMPLE 3

In this example we demonstrate a means by which the method of this invention could be used to produce a vaccine capable of eliciting an antibody response against Luteinizing Hormone Releasing Hormone (LHRH).

Mice are initially immunized against receptors for LHRH. After demonstrating that the resultant anti-LHRH receptor antibody responses include antibodies specific for the LHRH binding site expressed by the LHRH receptors, a cell fusion is performed to generate monoclonal antibodies which mimic the hormone (LHRH) and thus bind to the receptor's binding site. These monoclonal antibodies are identified by their capacity to interact with the site on the receptor to which LHRH binds. Next, the monoclonal antibodies are subjected to a second selection procedure to assess their

- 42 -

capacity to elicit antibodies that bind to LHRH. This step identifies the receptor specific monoclonal antibodies that can be used as an LHRH vaccine. Such antibodies may then be used as immunogens to evoke the formation of antibodies which resemble the receptor for LHRH and which thus bind to and neutralize the hormone.

#### I. Immunization Of Mice With LHRH Receptors

To immunize mice with LHRH receptors, an immunogen that contains the receptor binding site for LHRH is first prepared. This can be accomplished by several approaches. One approach is to utilize pituitary and gonadal tissues which contain cells that express functional receptors for LHRH, and can thus serve as sources from which the immunogen can be obtained. In this example, pituitary LHRH receptors are used as the immunogen. Ovarian receptors may, however, also be used.

There may be slight differences between pituitary and gonadal LHRH receptors with regard to their binding sites for LHRH. It may be preferable in particular applications to construct a bivalent vaccine containing antibodies specific for receptors from each of these sources. Such a bivalent vaccine would elicit a more heterogeneous immune response with regard to the specificities of antibodies against LHRH. It follows that the methods of this invention may be used to construct multivalent vaccines containing antibodies specific for

- 43 -

different receptors. In the case of pathogens that bind to more than one receptor type, a multivalent vaccine containing anti-receptor antibodies against each receptor targeted by a pathogen would induce immunity against each of the different ligands expressed by the pathogen, thereby enhancing the protective value of the vaccine.

The LHRH receptor preparation to be used as an immunogen in this example consists of purified receptors. Although it is desirable to obtain as pure a receptor preparation as possible, it is not required. For example, either dispersed pituitary cells and/or solubilized pituitary membrane preparations that contain the LHRH receptor could be used to induce anti-LHRH receptor antibodies. On the other hand, it would be most desirable to purify the receptor site that binds to the ligand and use the pure receptor as the immunogen.

Although the methods of obtaining the LHRH binding site from the LHRH receptor have not yet been developed, it has been hypothesized that the peptide encoded by the mRNA nucleotide sequence complementary to the mRNA that encodes LHRH resembles the LHRH binding site of the LHRH receptor. (Please see: J.J. Mulchahey, et al., "Antibodies to the Binding Site of the Receptor for Luteinizing Hormone-Releasing Hormone (LHRH) mRNA." Proc. Natl. Acad. Sci., USA, 83, pp. 9714-9718, 1986.) The complementary peptide could thus serve as an isolated LHRH receptor site, to be used to induce anti-LHRH receptor

- 44 -

antibodies when coupled to an immunogenic carrier. Indeed, should the complementary peptide approach apply to other ligand-receptor interactions, the complementary peptide approach could be broadly applicable as means to produce anti-receptor antibodies by the method of this invention.

We obtained LHRH receptors by purifying from bovine pituitaries according to the methods described by E. Hazum, et al., "Production and Characterization of Antibodies to Gonadotropin-Releasing Hormone Receptor", J. of Biological Chemistry, 262, pp. 531-534, 1986. These methods entail the preparation of bovine pituitary membranes by homogenization followed by differential centrifugation. The membrane preparations are next solubilized with detergent, then ultracentrifuged, and finally passed over an affinity chromatography column containing [biotinyl-D-Lys<sup>6</sup>] LHRH immobilized on avidin-agarose. The LHRH receptors are eluted from the column with a salt solution, dialyzed against physiological saline, concentrated by ultrafiltration, and finally assayed to determine their concentration.

The purified LHRH receptors are next shown to retain their LHRH binding capacity by the methods outlined by E. Hazum, et al., "Solubilization and Purification of Rat Pituitary Gonadotropin-Releasing Hormone Receptor", J. of Biological Chemistry, 261, pp. 13043-13048, 1986. According to this method, radiolabeled Buserelin (an agonist of LHRH) is

- 45 -

mixed with a sample of the LHRH receptor preparation. Unbound Buserelin is absorbed by the addition of activated charcoal. After removal of the charcoal, the quantity of receptor-bound radiolabeled Buserelin is determined by counting the supernatant in a suitable detector. The specificity of the binding is ascertained by inhibiting Buserelin binding with unlabeled LHRH. Only receptor preparations that retain the capacity to specifically bind LHRH are suitable for use as immunogens.

We next immunize 12 week old female CAF<sub>1</sub> mice with the purified receptor preparations. After obtaining pre-immune (normal) serum samples by tail vein bleeding, we inoculate each mouse intraperitoneally with 5-25 ug of purified LHRH receptors emulsified (1:1) in Freund's Complete Adjuvant (FCA) in a total volume of 0.2 ml. The mice are subsequently boosted at 3 week intervals with intraperitoneal injections of 5-25 ug purified LHRH receptors in 0.2 ml saline plus 0.1 ml of epinephrine, diluted 1:5,000 in saline. Two weeks after each injection, we obtain serum samples by tail vein bleeding.

## II. Assay For Serum Anti-LHRH Receptor Antibodies

In order to ascertain whether the mice are producing antibodies specific for the LHRH binding site on the receptor, we conduct an Enzyme-Linked Immunosorbent Assay ("ELISA"). In this assay, the antibody is labelled by a covalently attached



- 46 -

enzyme (instead of the radiolabel used in a radioimmunoassay). The enzyme attached to the antibody is one that can react with a colorless substrate to give a colored product. The amount of product released in a fixed period of time depends on the concentration of enzyme, and this in turn is a measure of the amount of antibody present. Spectrophotometric equipment reads the optical densities (O.D.), which correlates with the amount of bound antibody.

The assay is established as follows:

The target antigen in this assay consists preferably of rat pituitary LHRH receptors prepared as described by E. Hazum, "Solubilization and Purification of Rat Pituitary Gonadotropin-Releasing Hormone Receptor", J. of Biological Chemistry, 261, pp. 13043-13048, 1986. Less preferably, the target antigen consists of porcine pituitary LHRH receptors, purified as described above for bovine LHRH receptors. To conduct the ELISA, we coat Immulon II U-Plate (made by Dynatech) wells with 25 ul of a solution of LHRH receptors at 2 ug/ml in glycine coating buffer (0.1M, pH=9.5) and maintain the coated wells overnight at 4°C. The wells are rinsed four times with a wash solution consisting of saline to which we add 0.05% Tween-20 and 0.02%  $\text{NaN}_3$ . We then add 25 ul primary antibodies (tenfold serial dilutions of anti-LHRH receptor sera obtained from mice immunized with the LHRH receptor immunogen, running from  $10^{-1}$  through  $10^{-8}$  (or cell culture

- 47 -

supernatants when testing for monoclonal anti-LHRH receptor antibodies) per well and incubate for one hour at 22°C. All anti-sera dilutions are made in diluting buffer, consisting of FTA Hemagglutination Buffer with 0.05% Tween-20 and 0.02%  $\text{NaN}_3$ .

To verify antibody specificity, binding should be inhibited by first adding to each well 25 ul of LHRH (usually at 1.0 mg/ml) in diluting buffer before the addition of primary antibody. ELISA O.D. values from these wells are compared with O.D. values from wells to which 25 ul of diluting buffer alone has been added. We then rinse the wells again. We follow this with the addition of 25 ul of secondary antibodies per well, consisting of biotinylated rabbit anti-mouse Ig (1:1000 in diluting buffer) and incubate for one hour at 22°C. We again rinse the wells and then add to each well 25 ul of avidin-alkaline phosphatase conjugate (1:1000) and incubate for one hour at 22°C. We again rinse the wells, and we then add to each well 25 ul of 1 mg/ml p-nitrophenyl-phosphate (in 10% diethanolamine, 0.5mM  $\text{MgCl}_2$ , 0.02%  $\text{NaN}_3$ , pH=9.8) and allow the color to develop for 5-30 minutes. We determine the optical densities with a Dynatech Microelisa Reader. We coat other protein antigens, such as hen egg lysosome, bovine serum albumin, and bovine luteinizing hormone, as negative controls for antibody specificities onto plates and analyze them in a manner similar to that described above.

- 48 -

If LHRH is first allowed to bind to the LHRH receptor and thus occupy the receptor's LHRH binding site, antibodies specific for the LHRH binding site will subsequently be unable to bind. This LHRH dependant inhibition is indicative of the presence of binding site specific antibodies. The ELISA thus enables one to identify the mice that are producing the desired antibodies.

### III. Generation of Anti-Receptor Monoclonal Antibodies

Mice that are shown by ELISA to have produced antibodies specific for the LHRH binding site of the LHRH receptor are next utilized as spleen cell donors in cell fusions designed to generate anti-LHRH receptor monoclonal antibodies. The mice are boosted with interperitoneal injections of 5-25 ug of purified bovine pituitary LHRH receptors in 0.2 ml saline plus epinephrine (0.1 ml, diluted 1:5000 in saline). Three days later, cell fusions are performed according to the methods of B.B. Mishell and S.M. Shiigi [Selected Methods in Cellular Immunology. W.H. Freeman and Co., San Francisco (1980)]. The culture medium consists of RPMI 1640 (Gibco Laboratories) supplemented with 10% Fetal Calf Serum and 50 ug/ml gentamycin. We create hybridomas producing monoclonal anti-LHRH receptor antibodies by fusing spleen cells from LHRH receptor immunized mice with P3 tumor cells using polyethylene glycol. We select our hybrids by feeding on

- 49 -

Hypoxanthene-Aminopterin-Thymidine supplemented media and then screening our specific antibody using the ELISA protocol defined above. We consider cell lines to be established when their cloning efficiencies reached 100%. Samples of established cell lines are frozen and stored under liquid N<sub>2</sub>.

We generate working quantities of monoclonal antibodies as ascites tumors in the peritoneal cavities of mice and collect the ascites fluid, according to the methods of Mishell, supra. We inject CAF<sub>1</sub> mice with 0.5 ml of Pristane intraperitoneally. Three days later, we inject  $2 \times 10^6$  hybrid cells, suspended in 0.5 saline, into the mice intraperitoneally. After collecting the ascites fluid from the peritoneal cavity of mice, we centrifuge the fluid (400 x g for 10 minutes) to remove the cells.

#### IV. Identification Of Receptor-Specific Antibodies That Induce Antibodies Resembling The Receptor And Thus Bind LHRH

We next assess the capacity of the receptor specific monoclonal antibodies to elicit anti-LHRH antibodies in mice, as follows:

We render the receptor-specific monoclonals immunogenic for mice by coupling them to keyhole limpet hemocyanin (KLH). This saves us the necessity of using a different species to generate an antibody response to the monoclonals.

- 50 -

We isolate KLH, which is purchased as an ammonium sulphate haemolymph precipitate, by dialyzing the precipitate slurry against 0.5M NaCl followed by gel filtration over a Sephacryl 400 column (50 x 1.5 cm, 15 ml/hour, 1M NaCl). We pool KLH-containing fractions and determine the protein concentrations using standard spectrophotometric techniques with a Gilford Spectrophotometer 260 ( $A_{280}$  measurements). We then concentrate the combined material to 5.0 mg/ml on an Amicon concentrator.

In order to purify our monoclonal antibodies, we precipitate each ascites fluid sample with ammonium sulphate (40%), dialyze the precipitates against saline at 4°C, and then determine the protein concentrations by spectrophotometry. To prepare the conjugates, we dissolve 10.0 mg of each lyophilized antibody in 2.5 ml of 0.5 M NaCl containing 5.0 mg KLH. After centrifuging the mixtures to remove small amounts of insoluble material (10 minutes at 2000 x g), we add 50 mg of 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride to each mixture while stirring. The reactions are allowed to proceed for 16 hours at room temperature, then the mixtures are dialyzed against 0.5 M NaCl at 4°C. The dialyzed mixtures are stored frozen at -20°C and then thawed just prior to use.

We immunize mice with 100 ug of anti-receptor monoclonal-KLH conjugate emulsified 1:1 in Freund's Complete Adjuvant H37Ra (Difco). Each mouse is injected with conjugate that contains a single species of monoclonal anti-receptor

- 51 -

antibody. To do this we inject 0.1 ml of emulsion intraperitoneally in each mouse. The animals receive three additional intraperitoneal injections of 100 ug of antigen each, administered in alum at 21 day intervals. We obtain serum samples by tail vein bleedings 14 days after the fourth injection.

To identify the monoclonal antibodies that induce anti-LHRH responses, the pre-immune and immune sera are next assayed by ELISA for the presence of antibodies against LHRH. The ELISA is identical to that described for the LHRH receptor, except that as the target antigen we use LHRH conjugated to diphtheria toxoid (DT). To prepare the antigen, 3.0 mg of [glu<sup>1</sup>]-LHRH (Vega Biochemicals) is added to 15.0 mg of diphtheria toxoid (Connaught Laboratories) in 4 ml saline. 0.1 M NaOH is used to adjust the pH to 7.5. 19.2 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Pierce Chemical) is then added with the pH maintained at 7.5. The mixture is stirred for 8 hours at room temperature, then dialyzed at 4°C against saline. The LHRH-DT conjugate is stored at -20°C.

Serial dilutions of sera from each immunized mouse are allowed to react with LHRH-DT coating the wells of Microelisa plates. Each sample is reacted in the presence and in the absence of free LHRH. Sera containing LHRH specific antibodies are identifiable due to the specific inhibition of antibody binding by free LHRH. This, in turn, allows for the

- 52 -

identification of the monoclonal anti-LHRH receptor antibodies that were used as immunogens to elicit LHRH specific antibodies. Anti-receptor antibodies that mediate this effect can then be incorporated into a vaccine designed to induce antibody responses against LHRH.

While we have hereinabove presented a number of embodiments of this invention, it is apparent that our basic construction can be altered to provide other embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

- 53 -

We claim:

1. A method for making an immunogenic composition, said method comprising the steps of eliciting antibodies to the cell-receptor molecules specific for binding of a given agent, and selecting antibodies that immunochemically mimic said agent's receptor-specific binding site.
2. The method of claim 1, wherein the agent is a pathogen.
3. The method of claim 1, wherein the agent is a hormone.
4. The method of claim 1, wherein the agent is an enzyme.
5. The method of claim 1, wherein the agent is a toxin.
6. The method of claim 1, wherein the antibodies that immunochemically mimic said agents' receptor-specific binding site are monoclonal antibodies.
7. The method of claim 1, wherein the immunogenic composition is multivalent.



- 54 -

8. An immunogenic composition capable of eliciting antibodies to an agent's receptor-specific binding site, said immunogenic composition comprising an internal image of the agent's receptor-specific binding site.

9. The immunogenic composition of claim 8, wherein the agent is a pathogen.

10. The immunogenic composition of claim 8, wherein the agent is a hormone.

11. The immunogenic composition of claim 8, wherein the agent is an enzyme.

12. The immunogenic composition of claim 8, wherein the agent is a toxin.

13. The immunogenic composition of claim 8, wherein the immunogenic composition comprises monoclonal antibodies.

14. The immunogenic composition of claim 8, wherein the immunogenic composition is multivalent and comprises internal images of two or more of the agent's receptor-specific binding sites.

- 55 -

15. A method of immunization, consisting of administering an immunogenic composition comprising antibodies which immunochemically resemble an agent's receptor-binding site, said antibodies being capable of inducing the formation of antibodies that block the binding of the agent to the cell-receptor site.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01768

**I. CLASSIFICATION OF SUBJECT MATTER** (If several classification symbols apply, indicate all) <sup>3</sup>  
 According to International Patent Classification (IPC) or to both National Classification and IPC  
 INT CL4 A61K 39/395, 40, 42; C12P 21/00; C12N 15/00  
 US CL 424/85, 86, 87, 88, 89, 92; 435/68, 172.2; 530/387; 935/106, 107

**II. FIELDS SEARCHED**  
 Minimum Documentation Searched <sup>4</sup>  
 Classification System Classification Symbols  
 424/85, 86, 87, 88, 89, 92; 435/68, 172.2; 530/387, 808;  
 935/89, 106, 107, 108

US

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>16</sup> with Indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X	J. Biol. Chem., Vol. 258, Issued May 1983, "Monoclonal Antibodies to Receptors for Insulin and Somatomedin-C," (Kull), pages 6561-66, See pg. 6561.	1, 3, 6, 8, 10 13, 15
X	Proc. Natl. Acad. Sci., Vol. 77, Issued Dec. 1980, "Monoclonal Antibodies to $\beta$ -Adrenergic Receptors: Use in purification and Molecular Characterization of $\beta$ -Receptors," (Fraser), pages 7034-38, see pg. 7034.	1, 3, 6, 8, 10 13, 15
X	J. Cell. Biochem., Vol. 21, Issued 1983, "Immunological Studies of $\beta$ -Adrenergic Receptors," (Couraud) pg. 187-193, see pg. 187-88.	1, 3, 6, 8, 10 13, 15
X	PCT, A, WO85/02414, (Schochetman), June 1985, See pg. 5-6, 32-33	1, 4, 6-7
Y	Science, Vol. 228, Issued April, 1985, "Making Antibodies Without the Antigen", (Marx), pg. 162-65, see pg. 164	1-15

<sup>15</sup> Special categories of cited documents:

"A" document defining the general state of the art which is not  
considered to be of particular relevance

"E" earlier document but published on or after the international  
filing date

"L" document which may throw doubts on priority claim(s) or  
which is cited to establish the publication date of another  
citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or  
other means

"P" document published prior to the international filing date but  
later than the priority date claimed

"T" later document published after the international filing date  
or priority date and not in conflict with the application but  
cited to understand the principle or theory underlying the  
invention

"X" document of particular relevance; the claimed invention  
cannot be considered novel or cannot be considered to  
involve an inventive step

"Y" document of particular relevance; the claimed invention  
cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
in the art.

"G" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>1</sup>

23 September 1987

International Searching Authority <sup>1</sup>

ISA/US

Date of Mailing of this International Search Report <sup>2</sup>

15 OCT 1987

Signature of Authorized Officer <sup>19</sup>  
 Garnette D. Draper

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No <sup>18</sup>
Y	Fed. Proc., Vol. 43, Issued July 1984, "Monoclonal and anti-idiotypic Antibodies as probes for receptor structure and Function," (Venter) pg. 2532-39, see pg. 2533-37	1-15
Y	BioTechniques, Vol. 3, Issued 1985, "Vaccines Utilizing Internal Images Anti-idiotypic Antibodies that Mimic Antigens of Infectious Organism," (Kennedy), pg. 404,409, see pg. 405-08.	1-15
Y	Scientific American, Vol. 255, Issued 1986, "Anti-idiotypes and Immunity," (Kennedy), pg. 48-56, see entire document.	1-15
P,A	US,A, 4,661,586 (Levy), April 1987, see claims	1-15
A	US,A 4,490,358 (Greene), December 1984, See Abstract.	1-15
X Y	BioEssay, Vol. 3, Issued 1985, "Using Molecular Mimicry to Produce Anti-receptor Antibodies" (Linthicum), pg. 213-17. See all	1-4,6,8-11, 13 1-15